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Traits of Fluorescent *Pseudomonas* spp. Involved in Suppression of Plant Root Pathogens

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INTRODUCTION

Fluorescent *Pseudomonas* spp. make up a diverse group of bacteria that can generally be visually distinguished from other pseudomonads by their ability to produce a water-soluble yellow-green pigment. They are typically gram-negative, chemoheterotrophic motile rods with polar flagella and are grouped in rRNA homology group I, as defined by Palleroni et al. (132). This classification method divides all *Pseudomonas* spp. into five groups based on the relatedness of their rRNA genes, which undergo fewer changes than most other DNA sequences in the course of evolution. Fluorescent pseudomonads have simple nutritional requirements, and this is reflected by the relative abundance of these organisms in nature. They are found in soils, foliage, fresh water, sediments, and seawater, and the type species of the group, *Pseudomonas aeruginosa*, is a classified secondary pathogen of animals (166). As a group, the fluorescent pseudomonads are of primary significance in such diverse areas as medical pathogenicity, plant pathogenicity, food spoilage, and biological control. In this review, we will concentrate on the involvement of fluorescent pseudomonads in the biocontrol of plant root diseases.

Although some fluorescent pseudomonads, e.g., *Pseudomonas syringae*, are well-known plant pathogens, it is now generally recognized that members of this group can be beneficial to plants. During the last 25 years, research has illustrated the latent potential of exploiting certain bacteria for the biocontrol of root crop diseases. Beneficial or plant growth-promoting rhizobacteria have been isolated and demonstrated to protect the roots of certain root crop plants (19, 145). It is now recognized that some plant growth-promoting rhizobacteria may promote plant growth by secreting plant hormones (96). However, fluorescent *Pseudomonas* spp. have emerged as the largest and potentially most promising group of plant growth-promoting rhizobacteria involved in the biocontrol of plant diseases (86, 90, 156, 157). Other reported plant growth-promoting rhizobacteria include *Arthrobacter* (86, 150), *Alcaligenes* (173), *Serratia* (86), *Rhizobium* (23), *Agrobacterium* (84), and *Bacillus* (18, 112) spp.

Numerous examples of plant growth stimulation by fluorescent *Pseudomonas* species have been reported (11, 20, 52, 73, 82, 90, 155–157, 164, 170). These bacteria are ideally suited as soil inoculants because of their potential for rapid and aggressive colonization. This feature alone is suggested as a disease control mechanism by preventing the invasion of detrimental soil microorganisms onto the root surface (2). Significant increases in growth and yield of potatoes by up to 367% were reported in greenhouse experiments by Burr et al. (20) with specific fluorescent *Pseudomonas* strains. Similar growth and yield increases of potato by seed inoculation with fluorescent pseudomonads were obtained in field trials by Kloepper et al. (90) and by Geels et al. (55). Yield increases in sugar beet of 20 to 85%, due to disease control by fluorescent *Pseudomonas* spp., were obtained in greenhouse trials (157). Van Peer and Schippers (164) documented increases in root and shoot fresh weight for tomato, cucumber, lettuce, and potato as a result of bacterization with *Pseudomonas* strains. Fluorescent *Pseudomonas* spp. have also been implicated in the control of phytophthora root rot of soybean (97), tobacco black root rot (82), potato seed

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decay due to *Erwinia carotovora* (171), several wilt diseases due to *Fusarium* spp. (88, 142, 151), and fungal diseases of orange and lemon citrus roots (52) and ornamental plants (172).

The control of various *Pythium* fungi by specific fluorescent *Pseudomonas* strains has also been documented. Howell and Stipanovic (73) isolated a fluorescent pseudomonad which could inhibit *Pythium ultimum*, an important pathogen of cotton seedlings (50, 79). This strain was shown to increase seedling survival in *Pythium ultimum*-infested soil by up to 71% (73). *Pythium* species are also important pathogens of wheat, which is one of the world's major crops. At least 10 *Pythium* species may affect wheat (24) by infecting embryos and destroying root hairs and root tips (28). A number of fluorescent *Pseudomonas* strains which can increase the growth of wheat primarily by suppression of *Pythium* species have been isolated (12, 170). It has been reported by Weller and Cook (170) that a fluorescent *Pseudomonas* strain can increase wheat yields an average of 26% in field plots naturally infested with *Pythium* species. Significantly, increases obtained in greenhouse studies with this strain were equal to the increases obtained with the widely used commercial fungicide metalaxyl.

The above reports provide clear evidence of the phenomenon of plant growth stimulation by bacterial inoculants, and in particular they illustrate the realistic potential of exploiting specific fluorescent *Pseudomonas* strains for the biocontrol of major plant root diseases. It should be recognized, however, that there are problems preventing the commercial use of many of these biological control agents. Two major problems at present are the variable results obtained in different soil types (19, 145) and the inadequate survival of strains on seeds prior to planting (112, 155). Mutants resistant to some fungicides can be easily obtained (133), and so it has been suggested that the integration of present biological and chemical methods may be an improvement over the commercial control methods currently in use (7). It is recognized, however, that improvement of the biocontrol mechanisms of these bacteria by genetic or other means is an important approach to enhancing their performance as biocontrol agents (7). This strategy is a likely prerequisite if these strains are to be exploited commercially and routinely for plant root disease control.

PLANT DISEASE SUPPRESSION MECHANISMS

Siderophore-Mediated Suppression

The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Because almost all living organisms require iron for growth, survival in a heterogeneous environment such as the rhizosphere depends largely on the ability to scavenge sufficient iron from a limiting pool. To date, the only known exceptions to this rule are certain lactobacilli, which are devoid of heme proteins and hence have no iron requirement (4). The unavailability of iron for growth is surprising, since it is the fourth most abundant element in the Earth's crust (31). However, it is largely insoluble and thus is unavailable for direct microbial assimilation. In aqueous solution, iron can exist in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) form, the latter being the less soluble. However, in highly oxidized and aerated soils, the predominant form of iron is the ferric form (101), which is soluble in water (pH 7.4) at about 10^{-18} M (119). This is too low to support the growth of microorganisms, which generally need concentrations approaching 10^{-6} M for normal growth. Consequently, to survive in such environments, organisms secrete iron-binding ligands (siderophores), which can bind the ferric iron and make it available to the host microorganism. These compounds have been defined as "low-molecular-mass, virtually ferric specific ligands, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell" (117).

The structural diversity among the different siderophores is quite considerable and depends on the producing microorganism. However, a common feature of all siderophores is that they form six coordinate octahedral complexes with ferric iron (139). Most known siderophores can be grouped into hydroxamate- and phenolate/catecholate-type structures and have different affinities for ferric iron. Siderophores with the latter type of structure generally have higher formation constants with ferric iron, but the stability of these iron complexes is highly pH dependent (110). The hydroxamate complex is much more stable (117, 139) and hence potentially more significant in the rhizosphere.

Soil pseudomonads generally produce fluorescent, yellow-green, water-soluble siderophores with both a hydroxamate and phenolate group; these siderophores have been classified as either pyoverdins or pseudobactins. Analysis of pseudobactin- and pyoverdin-type siderophores from different fluorescent pseudomonad strains showed that the principal difference is the composition, number, and configuration of the amino acids in the peptide backbone (118). The production of these siderophores has been linked to the disease suppression ability of certain fluorescent *Pseudomonas* spp. (102). Research in the 1970s (2, 89) first suggested the involvement of these compounds in plant growth promotion and disease suppression by these strains. However, the first real substantiation of this concept was published in 1980 by Kloepper et al. (87), who isolated the fluorescent siderophore from strain B10 and showed that it mimicked the disease suppression ability of the producing strain. Furthermore, the inhibitory effects of both the purified siderophore and the producing strain were eliminated under high-iron conditions. Subsequent genetic evidence provided by a number of groups indicated that the inhibitory properties of certain fluorescent pseudomonads were abolished in siderophore-negative mutants (123, 163). More significantly, a Tn*5*-induced siderophore-negative mutant of fluorescent *Pseudomonas* sp. strain WCS358 lost the ability to promote the growth of potato (9). This report, published in 1986, was the first to demonstrate the involvement of fluorescent siderophores in plant growth stimulation under field conditions.

The proposed mechanism for siderophore-mediated disease suppression by fluorescent pseudomonads is illustrated in Fig. 1. Fluorescent siderophores, which have a very high affinity for ferric iron, are secreted during growth under low-iron conditions. The resulting ferric-siderophore complex is unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer cell membrane (21). In this way, fluorescent *Pseudomonas* strains may restrict the growth of deleterious bacteria and fungi at the plant root; this has been reviewed recently by Loper and Buyer (102). These iron starvation conditions may also prevent the germination of fungal spores. A direct correlation has been observed in vitro between siderophore synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of *Fusarium oxysporum* (42, 43, 151). This efficient iron uptake mechanism may also be a significant contributing

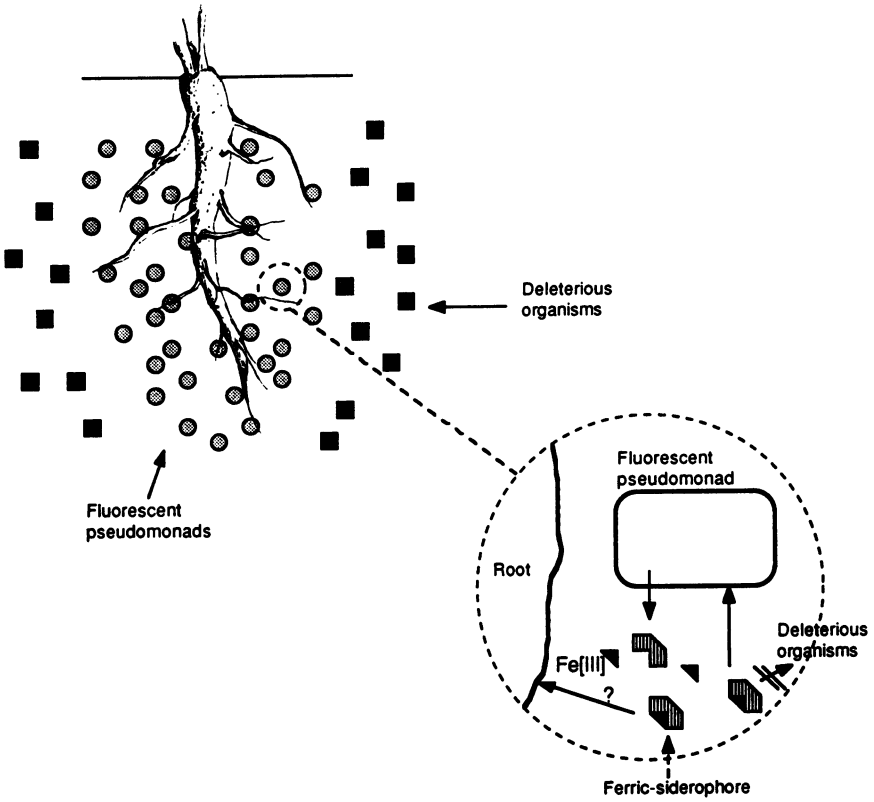

FIG. 1. Model for suppression of root pathogens by siderophores from fluorescent pseudomonads. The growth of deleterious organisms is restricted by the unavailability of the growth-limiting ferric iron. Adapted from references 21, 87, 88, 117, and 118.

factor to the ability of these strains to aggressively colonize plant roots, thus aiding the physical displacement of deleterious organisms.

Although iron competition among the rhizosphere population has attracted some research attention, it is not yet clear how this affects the iron requirements of plants. Iron deprivation in plants leads to a form of chlorosis (80). It has been reported that the fluorescent siderophore from *Pseudomonas* sp. strain B10 inhibited iron uptake by peas and maize plants (13). In contrast to this, there are a number of reports suggesting that plant species are capable of obtaining iron from some microbial siderophores (124, 138, 154). Cline et al. (25–27) demonstrated that iron from microbial hydroxamate siderophores may become available to plants both in nutrient solution and in soil. Furthermore, siderophores from fluorescent pseudomonads have also been implicated in iron uptake by tomato plants (41) and in the remedy of lime-induced chlorosis by peanut (81). Recent work has indicated that carnations and barley may utilize the fluorescent siderophore pseudobactin-358 for uptake of iron (40). These reports suggest that some plant species may obtain iron via certain microbial siderophores. It is also possible that plants are not able to utilize certain ferric-siderophore complexes. However, even these siderophores may not cause serious harm, because some plants can grow normally at much lower iron concentrations (10^{-9} M) (146) than those needed by bacteria (10^{-6} M). This may be due to superior iron storage mechanisms in higher organisms or the efficiency with which a given plant can acquire iron.

Siderophore production by fluorescent pseudomonads is influenced by a great variety of factors, e.g., concentration of iron (87); nature and concentration of carbon and nitrogen sources (59, 134); level of phosphates (10); pH and light (60); degree of aeration (94); presence of trace elements such as magnesium (57), zinc (22) or molybdenum (95); and temperature (168). Therefore, some authors, e.g., Misaghi et al. (114), suggest that siderophores may not be produced in sufficient quantities in soil to have any significant biocontrol effect. The examples discussed above however, suggest that siderophores are produced in some soils at sufficient quantities to suppress disease organisms. Furthermore, siderophores have been isolated from different soils (137). Geels and Schippers (56) found that siderophore-producing *Pseudomonas* strains were more effective at protecting potato crops from disease than some antibiotic-producing strains were. However, the amount of siderophore produced in soil could be significantly reduced because of all the factors regulating its production, and this must be variable depending on the soil type. Consequently, the ability to increase siderophore production by derepressing the regulatory mechanism may increase the biocontrol ability of fluorescent pseudomonads in a wider range of soil types. In our laboratory, we have addressed this problem by isolating a mutant of fluorescent *Pseudomonas* sp. strain M114; this mutant could produce siderophore in the presence of iron (128). It demonstrated increased in vitro bacterial and fungal inhibitory properties compared with the wild type, suggesting that this type of mutation may contribute to producing improved biocontrol strains (129). However, we have yet to evaluate this concept at the rhizosphere level.

Under favourable conditions, fluorescent pseudomonads can produce siderophores to >50% of the total dry weight of

the cells (113). Such values have led Newkirk and Hulcher (120) to state that "it seems unlikely that so much of the metabolic effort of *Pseudomonas mildenbergii* would be directed toward the accumulation of these substances (pyoverdines) if there was no biological function for them." Because soil is the natural habitat for fluorescent pseudomonads, it would make very little evolutionary sense if siderophores were not beneficial to these microorganisms in some soils.

Antibiotic-Mediated Suppression

Antibiotic production by some fluorescent *Pseudomonas* spp. is now recognized as an important factor in the disease suppression ability of these strains. The diversity in the type of antibiotics produced by different strains is only now being fully realised. Compounds such as phenazines (160), pyoluteorin (73), pyrrolnitrin (72), tropolone (98), pyocyanin (32), and 2,4-diacetylphloroglucinol (83, 147) have all been isolated from soil fluorescent pseudomonads. Some of these compounds, e.g., tropolone, have a broad spectrum of activity against many bacteria and fungi (98). Such an indiscriminate antibiotic agent may not always be desirable, because many beneficial organisms could also be eliminated. Other antibiotics exhibit different degrees of specificity and therefore may be more selective against certain microbially induced diseases. Howell and Stipanovic found that pyoluteorin from a fluorescent pseudomonad was an effective treatment for the protection of cotton seedlings from *Pythium*-induced damping off (73), whereas pyrrolnitrin from another pseudomonad was more effective for the protection of cotton seedlings from infection by *Rhizoctonia solani* (72). Until recently there was no direct evidence that the production of antibiotics in vivo contributed to the biocontrol ability of fluorescent pseudomonads. Typically, a correlation was made between the inhibition of pathogens by the production of antibiotics in vitro and the in vivo protection of plants from disease by selected strains. There are numerous reports of such cases in which control of specific root diseases is correlated with the in vitro production of an antibiotic inhibitory to the particular pathogen (32, 72, 73, 75). These observations were substantiated by genetic means, whereby mutants defective in the production of certain antibiotics were directly compared with their otherwise isogenic wild-type parental strains. Initial reports showed that antibiotic-negative mutants had lost the ability to inhibit specific pathogenic fungi (58, 65). Thomashow and Weller (160) went a step further and tested such mutants in vivo. It had previously been demonstrated (17, 62) that the fluorescent pseudomonad antibiotic phenazine-1-carboxylate was active in vitro against *Gaeumannomyces graminis* var. *tritici*, the causative agent of take-all disease in wheat. The mutational work by Thomashow and Weller (160) resulted in the isolation of a Tn5-induced mutant strain defective in the production of this phenazine antibiotic. These authors also found that as well as lacking in vitro inhibition of *G. graminis* var. *tritici*, the phenazine-deficient mutant exhibited greatly reduced protection of wheat from take-all compared with the isogenic wild-type strain. Confirmation of this genetic evidence for the direct involvement of an antibiotic in the biocontrol of a plant root disease was recently provided by Thomashow et al. (161). Using a high-performance liquid chromatography-based assay, they detected the phenazine antibiotic in the rhizosphere of wheat roots colonized by the phenazine-producing fluorescent pseudomonad. However, with the same assay, they detected no phenazine in the rhizosphere of wheat roots colonized by the phenazine-negative mutant. This report represented the first direct evidence for the production of an antibiotic by a fluorescent pseudomonad in the rhizosphere and its involvement in the control of a specific disease.

In our laboratory (147), the antibiotic 2,4-diacetylphloroglucinol (DAPG) was purified from a fluorescent *Pseudomonas* strain. Genetic evidence indicated that this compound could protect sugar beet roots from infection by *Pythium* fungi, since a Tn5-induced DAPG mutant had lost this ability. Furthermore, when tested in soil, this mutant showed a significant reduction in the emergence of sugar beet seeds compared with the yield in the presence of its isogenic parent (122). The same antibiotic compound produced by another fluorescent pseudomonad was found by Keel et al. (83) to suppress black rot in tobacco and take-all of wheat. These authors used a similar genetic approach to verify the involvement of DAPG in these systems. In a recent report (66), the same group also detected DAPG in the rhizosphere following colonization by DAPG-producing strains but not by the isogenic DAPG mutants. Consequently, the production of DAPG by fluorescent pseudomonads can occur in the rhizosphere, and its presence can be correlated with protection against specific diseases. Very little is known about the regulation of antibiotic compounds by fluorescent pseudomonads. However, the production of at least some antibiotics has been shown to be influenced by different physiological conditions. James and Gutterson (76) found that production of an antibiotic by *P. fluorescens* HV37a required the presence of glucose but that production of two other antibiotics by the same strain was inhibited by glucose. We have found a similar result in our laboratory with DAPG production from a fluorescent pseudomonad. Some sugars, e.g., sucrose, fructose, and mannitol, promoted high yields of DAPG, whereas glucose and sorbose resulted in very poor yields (147). DAPG production by this strain was also found to be influenced by temperature, with maximum production occurring at 12°C.

Other Mechanisms

HCN production. The production of hydrogen cyanide by certain fluorescent pseudomonads may also influence plant root pathogens. Voisard et al. (165) found that an HCN^- mutant (obtained by insertional inactivation) of the wild-type strain CHAO had lost its ability to suppress black root rot of tobacco. Subsequent work has shown that the same mutation had no effect on the biocontrol of take-all (*G. graminis* var. *tritici*) in wheat by this strain (66). Other workers, however, have found that HCN production by some fluorescent pseudomonads may in fact be detrimental to plant growth, since it has been implicated in the reduction of potato yields (8). Consequently, the role of HCN production by fluorescent pseudomonads in the control of root pathogens is as yet unclear. It is possible that HCN production in the rhizosphere has different effects on different plant types. At least for tobacco plants, it has been shown that HCN production by fluorescent pseudomonads stimulated root hair formation (165). This suggests that the biocontrol of HCN production by certain fluorescent pseudomonads may be due in part to the induction of plant resistance to certain pathogens.

Although there is no direct evidence for the involvement of fluorescent pseudomonads in plant-induced resistance, it has been postulated as a possible mechanism in some cases. For example, some strains have been shown to provide

biological control of *Pythium* or *Rhizoctonia* even though they did not exhibit any antagonism in vitro toward the pathogen (91, 96).

Competition. Although the production of siderophores and antibiotics has commanded the majority of research attention, it is generally recognized that there are other possible mechanisms in which fluorescent pseudomonads can protect plant roots from disease. Perhaps the most obvious, from a rhizosphere point of view, is the competition for nutrients and suitable niches on a root surface. Unless an organism can compete favorably with other organisms and effectively scavenge and utilize the available nutrients, it will not constitute a significant proportion of the rhizosphere-rhizoplane population (68). For an in-depth account of competition for nutrients and infection sites on a root surface, the reader is referred to a recent review by Paulitz (135). In the context of this discussion, only some major concepts will be addressed.

Nutrient competition varies at different rhizospheres depending on the available sources of carbon, nitrogen, sulfur, phosphate, and micronutrients. It is not yet clear whether a superior ability to utilize a particular type of nutrient or nutrients can confer a significant competitive advantage on a fluorescent *Pseudomonas* strain. In addressing this question, our laboratory has compared the rhizosphere performance of wild-type and isogenic mutants defective in specific nutritional capabilities. We obtained mutants of fluorescent *Pseudomonas* sp. strain M114 which were defective in protease production and the Entner-Doudoroff pathway (unable to catabolize glucose, trehalose, gluconate, fructose, mannitol, and glycerol) and had catabolic defects in succinate, malate, and fumarate utilization (131). In each case, no difference in the colonization potential between each mutant in direct competition with the wild type was detected under the assay conditions used. However, because in vivo soil conditions are very different, it remains to be determined whether these defects could have any significant effect on the plant-bacterium interaction in natural soils. Nevertheless, these experiments do suggest that at least in the sugar beet rhizosphere, there is a large variety of different carbon nutrient sources available which strain M114 can adequately use.

The concept of direct competition for available habitable niches on a root surface has not yet been clearly demonstrated for rhizosphere fluorescent pseudomonads. However, a very elegant example of this concept has been shown for a leaf-colonizing *P. syringae* strain. Ice nucleation-active (Ice^+) pathovars of *P. syringae* can cause frost damage to some plants by contributing to the formation of bacterial ice nuclei in the plant tissue at temperatures below freezing (5, 61). Lindow (99) constructed an Ice^- strain of an Ice^+ pathovar and showed that it could compete adequately with the wild type on a leaf surface. Because the leaf surface contains only a finite number of habitable sites, this had the effect of reducing the total number of Ice^+ pathovars on the leaf, thus decreasing the incidence of frost damage. The competitive exclusion of deleterious organisms by fluorescent pseudomonads at the plant root (Fig. 1) may also be a significant suppressive trait of these biocontrol strains. This mechanism has been suggested to play a role in the biocontrol by fluorescent *Pseudomonas* spp. of *Fusarium* (43) and *Pythium* (44) species.

Successful root colonization. The competitive exclusion of deleterious rhizosphere organisms is directly linked to an ability to successfully colonize a root surface. In effect, all disease-suppressive mechanisms exhibited by fluorescent pseudomonads are essentially of no real value unless these bacteria can successfully establish themselves at the root environment. It is well known that different fluorescent pseudomonads have different abilities to colonize a particular root niche (38, 90, 103, 153, 164). Many studies have been and are being done to determine what constitutes a good colonizing strain. This question has not yet been answered, but it is increasingly evident that a large number of integrative features are necessary for this process. This complex area has been reviewed extensively elsewhere (36, 93, 169), and in the context of this review, only a limited number of features will be highlighted to illustrate the difficulties encountered in assessing root colonization by pseudomonads.

When colonizing a root environment, an organism is confronted with a complex array of parameters such as water content, temperature, pH, soil types, composition of root exudates, mineral content, and other microorganisms. Numerous studies have been conducted to assess the contribution of each of these parameters. For example, the colonization of a fluorescent *Pseudomonas* strain in the potato rhizosphere was reported to be 10-fold greater in a sandy loam soil than in a clay loam soil (6) and another strain performed better in a sandy soil than in a peat soil (90). Although these experiments suggest that soil texture may have a direct influence on the colonization of these strains, other indirect factors associated with these soil types could also provide underlying reasons for the differences in colonization. Consequently, it is very difficult to determine the precise contribution of soil type on pseudomonad root colonization. The effects of moisture (74, 92, 106), temperature (103), and pH (29, 172) have also been investigated, and although the results do indicate some influences, it is difficult to ascertain whether the colonization changes observed are all due to direct effects of the parameters on the bacteria or to indirect effects on other rhizosphere components.

The potential of certain fluorescent pseudomonads to colonize a particular rhizosphere may also be hindered by their susceptibility to attack by other organisms. Protozoa are known to feed on pseudomonads, and this has been suggested to influence colonization potential in some instances (1). If this predation was not random but was confined to certain strains, protozoan predation could be an important factor to be considered when choosing biocontrol strains. However, the specificity of protozoan predation has not yet been addressed. *Bdellovibrio* sp., an obligate parasite of some soil gram-negative bacteria, has also been suggested to influence the survival of a pseudomonad in a soybean rhizosphere (144). However, it is also not known whether this parasitism is random or specific among the different fluorescent *Pseudomonas* strains. Work from our laboratory examined the effect of cell lysis by bacteriophages on colonization by fluorescent pseudomonads (153). This study found that numerous bacteriophages exist in soil and attack different *Pseudomonas* strains. Consequently, the colonization potential of a susceptible strain is reduced in the presence of a particular phage, but a resistant strain is unaffected. It can therefore be concluded that the variations in colonization potential exhibited by different strains of fluorescent pseudomonads can be due in part to specific cell killing by other biological entities.

By observing differences between good and poor colonizing strains, certain traits have been postulated to be significant for efficient colonization. For example, strain WCS365 (good colonizer) was shown to adhere in larger numbers to sterile potato roots than other inferior colonizing strains did, suggesting that attachment to the root surface enhances

colonization (36). Other studies have also obtained evidence suggesting a role for root attachment in pseudomonad colonization (3, 77). Differences in colonization between a fluorescent pseudomonad and isogenic flagella mutants prompted the conclusion that flagella are required for colonization of potato roots (38). However, in separate studies, other workers found no differences in the colonization of fluorescent pseudomonads and flagella mutants of these strains (74, 143). Taken together, these studies suggest that under certain conditions flagella may be required for good colonization, but under different circumstances they may have minimal importance. This illustrates the difficulties encountered in precisely determining the contribution of different traits for efficient colonization. At present, a good colonizing strain is determined by testing its performance in vivo. As more knowledge on the traits needed for efficient colonization becomes available, it may be possible to select strains with defined characteristics. This process would enable many more strains to be screened and also allow the possibility of improving these traits and thus enhancing colonization.

Introduction of new traits into strains. With the advent of genetic engineering, it is now clear that many more traits (not normally present in fluorescent pseudomonads) can be introduced into desired strains to enable them to combat pathogens more effectively. For example, the delta endotoxin from *Bacillus thuringiensis* has been stably inserted into the chromosome of a fluorescent pseudomonad from corn roots (121). The resulting recombinant strain was then able to inhibit certain insects. A gene encoding chitinase production from *Serratia marcescens* has also been cloned and expressed in a soil fluorescent pseudomonad (49). Chitinase degrades chitin, which is an inherent part of many plant-deleterious fungi and insects. Consequently, the introduction of this property into a desirable fluorescent pseudomonad could impart a new biocontrol property onto the strain. Gill and Warren (58) introduced a genetic region required for the production of a fungistatic antibiotic from one pseudomonad strain into another, imparting on the recipient strain an increased ability to inhibit fungi in vitro. The effectiveness of such genetically engineered strains under rhizosphere conditions has not yet been reported.

GENETIC ANALYSIS OF IMPORTANT TRAITS IN BIOCONTROL

Siderophore Production

The cloning of a group of genes required for fluorescent siderophore production in *Pseudomonas* sp. strain B10 by Moores et al. (115) in 1984 illustrated the complexity of the gene system required for this process. Using a mutant complementation analysis, these authors suggested that a minimum of 12 genes arranged in four gene clusters was required for the biosynthesis of pseudobactin by this strain. Workers in our laboratory (126) and others (104, 108) have also found a similar level of complexity in the arrangement of genes encoding biosynthesis of pseudobactin-like siderophores in other rhizosphere fluorescent pseudomonads. However, the biosynthetic pathway for pseudobactin siderophores is as yet undetermined, and consequently the function of any of the cloned biosynthetic genes has not yet been elucidated. This is in contrast to the well-known siderophores of *Escherichia coli* (aerobactin and enterobactin), whose biosynthetic pathways and corresponding genetic regions are now well established. Unlike these siderophores, however, the pseudobactins have a relatively complex structure, which may account for the large number of genes involved in their biosynthesis.

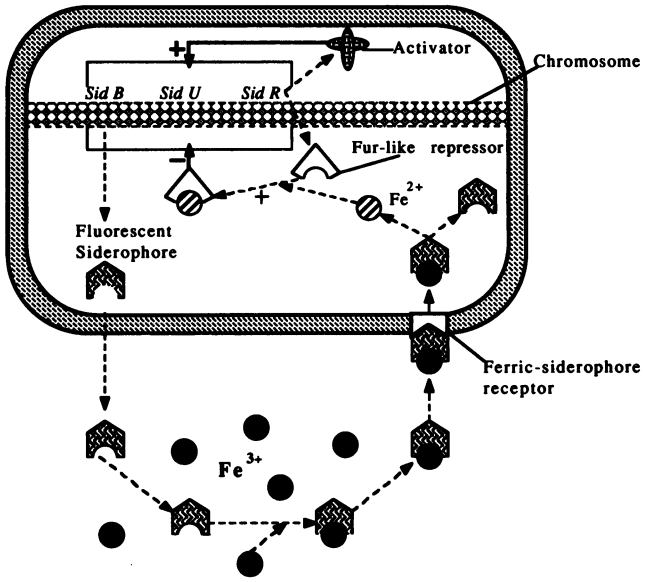

FIG. 2. Proposed model for high-affinity iron uptake in fluorescent pseudomonads as deduced from results presented in references 25, 44, 113, 116, 118, 119, 125, 137, 139, and 141. The mechanism of repressor-mediated regulation in pseudomonads has not yet been elucidated but probably involves metal activation, as in the *E. coli* Fur system (41). *Sid B*, siderophore biosynthetic genes; *Sid U*, ferric-siderophore uptake genes; *Sid R*, siderophore regulatory genes.

Ferric-pseudobactin uptake. Most of the research attention in the pseudobactin system in fluorescent pseudomonads has been focused on ferric-pseudobactin uptake and its regulation. This work has greatly increased our understanding of iron uptake in these bacteria, as illustrated by the schematic representation in Fig. 2. Ferric-pseudobactin uptake is mediated via a specific outer membrane receptor. The structural diversity of the different pseudobactin siderophores is also reflected in their cognate receptors, which generally exhibit a high degree of specificity (71). The molecular mass of these receptors is usually between 75 and 90 kDa. Ferric-pseudobactin receptors have been cloned by using a strategy which exploited the specific nature of the receptor and the relatively nonspecific nature of iron uptake proteins in the periplasm and cytoplasmic membrane (107, 109, 126). This involved the use of a wild-type *Pseudomonas* strain which could not utilize ferric-siderophore produced by the strain of interest. Siderophore obtained from the producing strain and incorporated in a low-iron medium would therefore inhibit its growth. However, by introducing a gene bank of the producing strain, clones encoding the receptor were selected because they enabled the wild-type strain to use the ferric-siderophore in the medium, thus reversing its inhibitory properties.

Characterization of cloned receptors has confirmed that they are required for the initial binding of the ferric-siderophore complex. In our laboratory, it has been shown that this event occurs almost immediately and is absolutely dependent on the presence of the correct receptor (116). The receptor enabling uptake of pseudobactin 358 has also been cloned (109) and has been shown to be extremely specific for

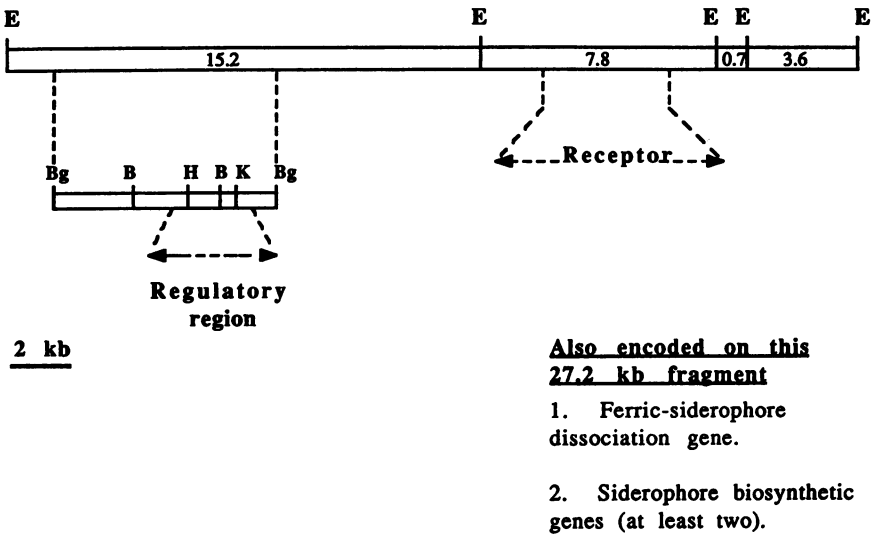

FIG. 3. Clustering of ferric-siderophore uptake genes with siderophore biosynthetic and regulatory genes in strain M114. The precise positions of the biosynthetic and dissociation genes were not determined but were localized to this fragment by a mutant-complementation analysis (137). Abbreviations: E, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I.

this siderophore. However, strain 358 has been shown to utilize other ferric-pseudobactins, and this uptake is probably mediated through other receptors (15). This theory of multiple receptors in fluorescent pseudomonads for uptake of different ferric-siderophores was first suggested by Buyer and Leong (21). These authors found that the fluorescent siderophore from strain A214 could not be utilized by strain B10 but that pseudobactin B10 could be utilized by strain A214. The existence of a second receptor in strain M114, which enables the strain to use another fluorescent siderophore from strain MT3A, was recently confirmed (116). The gene encoding this second receptor was cloned and was found to encode an 81-kDa outer membrane protein, differing somewhat from the resident receptor enabling uptake of M114 ferric-siderophore, which is 89 kDa (126). Virtually all fluorescent pseudomonads examined contain a number of iron-regulated proteins in their outer membrane that are compatible in size with ferric-pseudobactin receptors (37, 71). This would suggest that most strains may harbor additional receptors specific for the uptake of siderophores produced by other fluorescent pseudomonads. Since we now know that wild-type rhizosphere strains can accumulate additional receptors, it strongly suggests that there is selective pressure in their habitat for this event to occur. If this is the case, engineering of strains to incorporate additional receptors may improve their performance as biocontrol agents in the rhizosphere. Little is known about the fate of the ferric-pseudobactin complex following binding to the receptor. Our work has indicated (126) that the complex may be taken into the cytosol, with the iron being released from the siderophore, most probably by a reductive mechanism. This was suggested by the isolation of a mutant of strain M114, which accumulated M114 ferric-siderophore in the cytosol but had very low levels of Fe^{2+} compared with the wild type. Ferric iron reductases have previously been detected in most bacteria including *Pseudomonas* species (30), and because siderophores have very little affinity for the divalent metal, the above mechanism is thought to be the universal method for the dissociation of these complexes. A recent publication on the sequence of the receptor for pseudobactin 358 (15) has indicated that it contained regions of homology to the binding site of the *E. coli* TonB protein. This protein is involved in the coupling of the inner and outer membranes during transport across the cell envelope and is required for all ferric-siderophore transport systems in *E. coli* (136). It is therefore possible that the ferric-pseudobactin complex crosses the *Pseudomonas* cell envelope in a similar fashion prior to the internal reductive release of iron.

Because of the lack of a detailed linkage map for the chromosome of fluorescent pseudomonads, the location of the genes encoding the siderophore system is not known. However, there does appear to be a large degree of clustering of siderophore biosynthetic, uptake, and regulatory genes. The receptors for uptake of ferric-siderophores from strains M114 (126), B10 (107), and WCS358 (109) have all been cloned and shown to be closely associated with siderophore biosynthetic genes, albeit on different operons. Results from our laboratory (126) have shown that the M114 receptor is also clustered with a ferric siderophore dissociation gene and a locus encoding a negative regulator of iron uptake genes (Fig. 3). The gene encoding the second receptor in strain M114 (for uptake of MT3A ferric-siderophore) was not found to be clustered with any other iron uptake genes (116).

Iron regulation. The repressive effect of iron on siderophore production was first recognized in 1955 by Garibaldi and Neilands (53, 54). This discovery was instrumental in the detection and isolation of siderophores from most microbial species. However, it was much later before the mechanism of this "iron effect" was elucidated. This mechanism, designated Fur (ferric uptake regulation), was first proposed in 1978 by Ernst et al. (46) for the control of siderophore-mediated iron uptake by *Salmonella typhimurium*. A Fur repressor system was identified in *E. coli* in 1981 (67). Unlike the situation for *S. typhimurium*, for which a *fur* mutant was accidentally obtained by a fortuitous secondary mutation in the course of other mutation work by Lopes et al. (105), a *fur* mutant of *E. coli* was identified by means of a convenient screening procedure exploiting gene fusion technology. In this work, the promoter for expression of the *fhuA* gene

(encoding ferrichrome receptor) was fused to the *lacZ* reporter gene. The resulting iron-regulated construct was then introduced into a mutant bank of *E. coli* cells, and a strain which exhibited derepression of the construct was selected (67). By constructing a pseudomonad iron-regulated promoter-*lacZ* fusion construct, a *fur*-like mutant of strain M114 was isolated in a similar fashion (128). Characterization of this mutant revealed that it had the expected phenotype of a *fur*-like regulatory mutant since (i) multiple pseudomonad iron-regulated promoter-*lacZ* fusion constructs (including some derived from another strain WCS358) were deregulated in this strain, (ii) no defect in iron uptake was observed, and (iii) production of both siderophore biosynthetic and some uptake proteins was not regulated by iron (128). It was noted, however, that one of the outer membrane iron-regulated proteins was not fully derepressed in this mutant. A similar finding was observed for an *E. coli fur* mutant in which the outer membrane receptor protein for ferric-enterobactin (FepA) was not fully derepressed and for which an additional control mechanism has been suggested (67). This additional control may serve as a protective mechanism so that the uptake of iron may be stringently controlled to prevent iron toxicity within the cell.

Exploitation of siderophore-derepressed mutants may have potential implications in the area of improvement of biocontrol. The deregulated mutant of strain M114 was shown to exhibit inhibition of both bacterial and fungal indicator organisms under conditions in which the iron concentration was sufficiently high to render the wild-type strain ineffective (128). This suggests that the performance of certain biocontrol fluorescent pseudomonads may be improved by manipulation of their repressor systems.

The Fur repressor from *E. coli* has been purified (167) and has been shown to bind the promoter region of iron-regulated promoters (33), thus blocking transcription. However, prior to binding, it is activated by complexing with a divalent metal (33). This is in contrast to the mechanism of iron regulation in eukaryotic cells, which occurs at the translational level; i.e., a repressor protein (IRE-BP) binds the mRNA, thus preventing translation (140). Recent work from our laboratory has indicated that the *E. coli* Fur protein may be capable of repressing expression from fluorescent pseudomonad iron-regulated promoters and that this repression occurs at the transcriptional level (130). Sequence analysis of one promoter (SP1) had indicated that it contained regions homologous to the *E. coli* Fur-binding site. In addition, following expression of SP1 in *E. coli*, it was found to be iron regulated (130). It has also been demonstrated in vitro by protein–DNA-binding assays and DNA footprinting that the *E. coli* Fur protein can bind to a DNA fragment containing this fluorescent pseudomonad iron-regulated promoter at a specific site upstream from the start of transcription (125). This work indicated that the mechanism of Fur regulation on this iron-regulated promoter from strain M114 is to sterically impede the binding of factors needed for transcription initiation. Furthermore, because a *fur*-like mutant of strain M114 which exhibits deregulated expression from this promoter had previously been isolated, it is most likely that a repressor similar to Fur is present in *Pseudomonas* sp. strain M114.

Expression of pseudomonad iron-regulated promoters in *E. coli* does not normally occur, because the *E. coli* transcription machinery is not adequate for this purpose. A deletion analysis upstream of the SP1 promoter has indicated that >77 but <131 bp was required for promoter expression (130). This strongly indicates that such promoters require an activator for transcriptional initiation and hence may explain why pseudomonad iron-regulated promoters are not expressed in *E. coli*. Consequently, to express the SP1 promoter in *E. coli*, the *trans*-acting factor was cloned from *Pseudomonas* spp. and introduced into *E. coli*, thus allowing promoter expression to occur. Although transcriptional activators are very common for gene expression in *Pseudomonas* spp., including the iron-regulated *P. aeruginosa* genes encoding exotoxin A (69, 70) and elastase (51), this work represented the first indication of positive regulation of soil pseudomonad iron-regulated genes. Positive regulation of genes encoding the biosynthesis of the anguibactin siderophore of *Vibrio anguillarum* has already been established (162). This system requires a *trans*-acting factor (162) and a *cis*-acting factor (141) functioning cooperatively for optimum expression of its siderophore genes. However, a start has just been made to understand iron regulation in *Pseudomonas* spp., and further investigation will be necessary to elucidate the intricacies of the mechanisms of activator-repressor regulation.

Antibiotic Production

Antibiotic production has recently been recognized as an important feature in the biological control of plant diseases by fluorescent pseudomonads, and a number of researchers are now investigating the genetics of these compounds. The first report on the cloning of antibiotic genes was published in 1986 by Gutterson et al. (65). This work outlined the isolation of mutants of *P. fluorescens* HV37a which had lost the ability to inhibit *Pythium ultimum* on iron-rich media. It is generally assumed that the inhibition of test organisms on iron-rich media by fluorescent pseudomonads is due to the production of antibiotic-like substances other than siderophores. Complementation analysis of these mutants indicated that at least five genes in this strain encoded fungus-inhibitory substances. It was subsequently determined by thin-layer chromatography analysis that strain HV37a produced at least three different antibiotics (76). One of these compounds has been designated oomycin A and was found to be responsible for ~70% of the ability of this strain to reduce *Pythium* root infection of cotton and ~50% of its ability to increase cotton seed emergence (75). The production of these compounds was found to be regulated differentially by glucose, since production of one required the presence of glucose whereas production of the other two was inhibited by glucose (76). This regulation occurs at the transcriptional level and is dependent on the products of the *afuA* and *afuB* genes (64). Four transcriptional units which were involved in antibiotic biosynthesis were identified in this strain and designated *afuE*, *afuR*, *afuAB*, and *afuP* (64). The *afuAB* operon is involved in regulation, but the precise functions of the other units are not yet clear. The regulation of the *afuE* transcriptional unit appears to be more complex than the others, because in addition to catabolite induction by glucose, it appears to be autoregulated (64). Expression of *afuE* has been increased by cloning it downstream from the *tac* promoter, and this resulted in increased production of oomycin A by the engineered strain (63). It is thought that the inconsistencies observed in the in vivo biocontrol performances of fluorescent pseudomonads may be due in large part to a reduction in the amount of antibiotic produced under different environmental conditions (159). Consequently, altering its regulation may improve the performance of the pseudomonads in the rhizosphere. Preliminary experiments on the oomycin A-overproducing strain suggest an

improved inhibitory capacity against *Pythium ultimum* in vivo, compared with that of the parent (63).

Another antibiotic active against *Pythium ultimum* was found to be iron antagonized and iron regulated, as are siderophores (58). This study suggested that the antibiotic was not biochemically or genetically related to the pseudobactin-like siderophore of this strain. The production of compounds which are not involved in iron nutrition but are iron regulated has also been observed in *P. aeruginosa* (16) and several other systems (47). These observations have led to the suggestion that iron may act as a genetic signal for the expression of diverse functions which may be required during growth in low-iron habitats (47).

Phenazines. Genes involved in phenazine production in the fluorescent biocontrol strain 2-79 have also been cloned, but very little is known about their function (160). A 20.4-kb DNA region was isolated from a closely related strain, 30–34, which complemented several phenazine mutants, and the structural genes were localized to within 5 kb by Tn5 mutagenesis and deletion mapping (159). However, little is known about the organization of the genes involved. A phenazine-*lacZ* fusion construct, which was chromosomally inserted in strain 30-34, was used to investigate the in vitro regulation of the synthesis of this compound (159). Glucose was found to inhibit production, as was ammonia. However another source of nitrogen, nitrate, was found to stimulate β-galactosidase production in this fusion strain. Iron was noted to induce expression five- to eightfold. This is in contrast to the production of another antibiotic-like compound in strain NZ130, which was found to be iron repressed (58).

2,4-Diacetylphloroglucinol. The genetics of DAPG production is currently very much in its infancy. The cloning of a 22-kb DNA fragment from strain CHAO which complemented DAPG mutants of this strain has been reported (66). However, the organization of the genes involved has not yet been elucidated. In our laboratory, a gene(s) involved in DAPG production in strain F113 has been localized to a 6-kb DNA fragment. Transfer of this fragment into wild-type strain M114 conferred on this strain the ability to produce DAPG (48). Since DAPG production by strain F113 has been shown to be a significant biocontrol characteristic (122), it is likely that this new trait will enhance the biocontrol characteristics of strain M114. However, this concept has not yet been tested in vivo. Introduction of the fragment into *E. coli*, however, did not result in detectable DAPG production, suggesting that the genes may not be expressed or that other intermediates needed for DAPG production which are present in strain M114 are absent from *E. coli*. Cloned phenazine genes were found not to be expressed in *E. coli* (159). However, this work does highlight the possibility of introducing new traits into selected strains with a view to improving their performance as biocontrol strains.

Colonization

The colonizing ability of a strain can at present be evaluated only in vivo. Sufficient information on the factors involved in this complex process is not yet available to enable an assessment to be made by investigating the biochemical or genetic makeup of a strain. However, through the use of genetic means, some factors which play a role in colonization of root surfaces are being recognized. Recent genetic work has shown that the ability of a strain to attach to a particular plant glycoprotein (agglutinin) was correlated with its colonization potential of a root system (3). Mutants defective in this ability (Agg^-) exhibited reduced colonization of a root system and also a corresponding reduction in protection of cucumber roots from *Fusarium* wilt suppression (158). Furthermore, Tn5-induced mutants which exhibited increased adherence to agglutinin were also obtained (3), suggesting that this property may be negatively regulated by the fluorescent pseudomonad. However, the organization of the gene(s) involved in agglutinin adherence or the nature of this putative regulatory system has yet to be elucidated.

Some information on the involvement of certain pseudomonad characteristics in root colonization has been obtained indirectly by inducing a mutation in particular traits and subsequently evaluating its effect on colonization. For example, by using this strategy, exoprotease production by a fluorescent pseudomonad was found to be nonessential for the colonization of sugar beet roots (131) and loss of the O-antigen liposaccharide side chain from strains WCS358 and WCS374 was not found to affect their colonization of potato roots under the conditions used (39). However, a more direct strategy for obtaining information on root colonization is to examine genes which are specifically expressed in the rhizosphere. The genetic tools necessary for this direct approach are now available, and over the next few years some insight into the nature of gene expression in the rhizosphere should be obtained. These tools depend mainly on reporter genes, e.g., *lacZ* (148), *lux* (111), *xylE* (174), and *gus* (78), which can be fused to various promoters for convenient and quantitative monitoring of expression. One approach is to clone promoters into suitable promoter probe plasmids (152) and evaluate their expression in different strains in the rhizosphere. This approach was recently used with the *lux* reporter genes which encode bioluminescence. Fluorescent pseudomonads harboring these constructs could then be detected in the rhizosphere, and in this study the authors concluded that the *lux*-based detection system was at least 10^3-fold more sensitive than β-galactosidase based systems (35). Alternatively, there are now in vivo delivery systems (127, 149) for the specific chromosomal insertion of a reporter gene into a transcriptional unit, leading to the construction of more stable fusion strains.

In vivo promoter-*lacZ* fusions in a fluorescent pseudomonad, which are induced by the presence of D-xylose and L-arabinose (major wheat root exudate compounds), were found to be expressed in the wheat rhizosphere (45). This work demonstrates how genes which are expressed in the rhizosphere can be identified by using this technology.

Another exciting use of gene fusion technology is for the evaluation of soil parameters. Expression of an in vivo promoter-*lacZ* fusion in a fluorescent pseudomonad, which was induced by phosphate limitation, has been used to evaluate rhizosphere phosphate concentration (34). By measuring *lacZ* expression from this fusion strain during growth in the wheat rhizosphere, these authors were able to correlate expression with the phosphate levels in the soil. Information on soil parameters is essential when selecting strains for biocontrol purposes in soil.

EVALUATION OF GENETICALLY MODIFIED ORGANISMS

Genetic studies on important biocontrol traits of fluorescent pseudomonads have shown that there is a latent potential to improve the versatility and performance of these strains for biocontrol purposes. For example, ferric-siderophore receptors have been cloned and introduced into new strains, allowing them to use a wider range of siderophores

for iron uptake purposes (21, 109, 126). Inactivation of the Fur-like repressor of a fluorescent pseudomonad has been shown to improve the inhibitory characteristics of the strains under high-iron conditions (128). Studies on antibiotic production have shown that transferring genes encoding antibiotic production from one strain to another can increase the range of pathogens inhibited by the modified strain (48, 58). Genetic modifications such as these can introduce or improve desirable traits in suitable organisms, and this is therefore an attractive option for the commercialization of these strains.

The potential risks that may be associated with the release of some genetically modified organisms (GMOs) into the environment are not fully predictable. This situation has led to the establishment of regulatory bodies, which provide guidelines for the release of GMOs. To date very few GMOs have been released. One successful example of a commercial GMO which has been tested and is now used for the control of crown gall on stone fruit trees is *Agrobacterium rhizogenes* K1026 (85). Examples such as this, as well as specific research programs to understand the fate and interactions of GMOs in the environment, help provide the information necessary to predict any possible risks which may have to be addressed prior to the release of a particular GMO. The risks associated with GMO release, however, depend on the specific modification as well as the type of organism used. An underlying objective is to understand which factors might influence the survival and spread of different wild-type microorganisms in the environment. This information is a prerequisite before one can evaluate the effect of a genetic alteration on the fate of a particular microorganism in soil. Another line of research is to devise GMOs which will self-destruct after a desired period (100). For example, the *hoc* gene, which encodes a protein that causes lethal collapse of the transmembrane potential of cells, has been introduced into strains under the control of the inducible *lac* promoter such that following induction the cells would die (14). Strategies such as these may prove useful in designing new strains which are destined for release into environments and whose proliferation can be limited.

CONCLUSIONS

The possibility of replacing chemical control of plant root diseases with a biological alternative remains an exciting and challenging objective. Some fluorescent *Pseudomonas* spp. do appear to be prime candidates for this role. Progress is now being made at understanding some of the traits of fluorescent pseudomonads which are necessary for this process. It is imperative that progress in this area be matched with progress in the improvement of strategies for the application of strains to seeds, which at present is inadequate for commercial conditions. There is good evidence from soil experiments for the involvement of siderophores, different antibiotics, and HCN in the biocontrol of plant root diseases by fluorescent pseudomonads. However, the integration of these traits with the ecological fitness of strains will be a prerequisite for designing biocontrol agents for specific purposes. In effect, the biocontrol of plant root pathogens by fluorescent pseudomonads is a multifaceted process. Consequently, it would be unwise to consider selecting biocontrol strains on the basis of just one mechanism. It is likely that optimum performance may require the cooperative action of a number of these mechanisms. A better understanding of root colonization and disease suppression mechanisms is needed before suitable strains can be directly selected and/or engineered by genetic means into commercially viable products. The commercialization of GMOs is at present influenced by the procedures and time required for obtaining permission to release them into the environment. However, studies on factors affecting root colonization, persistence, and spread of both wild-type and altered fluorescent pseudomonads in soil will increase our knowledge of potential risks and consequently facilitate the release process. It is often suggested that it may not be necessary to genetically modify fluorescent pseudomonads in the quest to develop commercial biocontrol agents. It has been argued that once the desired attributes are recognized, wild-type strains with these attributes can be directly selected. However, a major problem to date has been the inconsistency of the performance of strains as disease-suppressive agents. One possible reason for this is that organisms respond to different stimuli in their environment, which leads to an alteration in metabolic activities. It may be necessary to genetically modify these metabolic traits to improve the consistency of the performance of strains under different conditions. It is therefore vital to understand the mechanisms for root colonization and disease suppression and how these respond to different stimuli before the performance of strains can be predicted or improved for particular soil and climatic conditions. This information is emerging. Research along these lines will increase the impact of fluorescent pseudomonads on the biocontrol of plant root diseases in the commercial world.

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